

Effect of mixing allergenic extracts containing *Helminthosporium*, *D. farinae*, and cockroach with perennial ryegrass

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Allergenic extracts used for immunotherapy often contain mixes of different allergens. Studies have shown that certain allergenic extracts contain enzymes that can cause a decrease in grass pollen allergenicity when mixed with grass extracts.

Glycerin and buffered saline with phenol (BSP) extracts containing Helminthosporium interseminatum, D. farinae, and cockroach were mixed with perennial ryegrass extracts and analyzed 7, 30, 60, and 90 days after mixing to determine the mixing effect of these extracts on the ryegrass pollen potency. Analysis was performed using RAST inhibition, SDS-PAGE and, to determine biologic potency, a quantitative intradermal skin test technique. All tests showed significant decreases in ryegrass potency when mixed with Helminthosporium and cockroach. This decrease was not seen with the D. farinae mix. Glycerin seemed to have some protective effect. Even in the situation showing the most decrease in ryegrass potency (Helminthosporium mixed with ryegrass in a BSP extract), the resulting extract still contained an estimated biologic potency of 10,000 AU/mL. This may explain why such mixes, which have been used clinically for many years, appear to give adequate therapeutic results. The study also suggests that clinicians may be well advised not to mix grass pollen extracts with extracts that may contain proteolytic enzymes.

INTRODUCTION

Allergen extracts used for immunotherapy are often complex mixes of different materials. Recent studies have shown that extracts may contain enzymes that are capable of degrading proteins.¹ Since most important allergens are proteins, these enzymes have the potential to destroy important components of allergenic extracts.

Studies have shown that grass extracts, when mixed with the molds *Alternaria* or *Helminthosporium*, (even when buffered with saline containing human serum albumin as a diluent) exhibited significant decreases in activity as measured by ELISA inhibition and immunoblot test.² These decreases in activity were presumed to be due to enzyme degradation of major grass allergens.

In addition, studies have shown that fungal, epithelial, and insect sourced extracts when mixed with

pollen extracts can cause a reduction in the potency of the pollen extracts as measured by ELISA inhibition. The potency reduction was correlated with the protease activity measured in these nongrass extracts.³ Studies have reported that house dust mites and mite extracts contain protease activity⁴⁻⁶ as do cockroach extracts.⁷

The studies to date have been performed using RAST inhibition, ELISA inhibition, and other in vitro assays. The clinical relevance of these studies is not entirely clear. This study was designed to help determine the clinical relevance of proteolytic enzyme-induced decreased potency of grass pollen extracts. Specifically, the purpose of the study was to determine whether mixing a mold extract (*Helminthosporium interseminatum*), a mite extract (*Dermatophagoides farinae*), or a cockroach mix extract (American, German, and Oriental) with a perennial ryegrass extract would result in a significant decrease in potency of the ryegrass extract. Both glycerinated and aqueous (buffered

saline with phenol) extracts were used to determine whether different diluents affected the degradation process in different ways. The mixtures were analyzed by in vivo and in vitro methods 7, 30, 60, and 90 days after they were prepared. The study was thus designed to compare in vivo with in vitro methods of measuring extract potency. A quantitative intradermal skin test measurement assay was used to determine clinical relevancy. The extract mixes were assigned allergy units based on this skin testing method. The in vitro tests used to analyze the extract mixes were RAST inhibition and SDS-PAGE and HPLC.

METHODS AND MATERIALS

Subjects

Subjects had a history of allergic rhinitis and/or asthma, with a seasonal pattern of symptoms suggestive of grass pollen allergy. They exhibited a puncture induced summation of erythema diameter skin test reaction greater than 40 mm when tested with the perennial ryegrass

Received for publication December 21, 1992.

Accepted for publication March 17, 1993.

grass extract. All exhibited a negative puncture skin test to the control extract shagbark hickory. In addition, subjects tested with the ryegrass/*D. farinae* mixture exhibited a negative puncture test to *D. farinae*, those tested with ryegrass/*Helminthosporium* exhibited a negative puncture test to *Helminthosporium*, and those tested to ryegrass/cockroach exhibited a negative puncture test to cockroach. At each timepoint three subjects were skin tested to the ryegrass/*D. farinae* extract, three to the ryegrass/*Helminthosporium* extract, and three to the ryegrass/cockroach extract. All subjects were tested with the ryegrass/hickory mixture and the ryegrass/ABS controls.

Skin Tests

Subjects were skin tested to test and control extracts using the Center for Biological Evaluation and Research (CBER) approved Relative Potency Protocol.⁸ A local Institutional Review Board reviewed the protocol and informed consent was obtained from all subjects prior to testing. Skin testing was performed using test and control extracts at day 7, day 30, day 60, and day 90 after the mixtures were prepared.

Allergy units were derived from D_{50s} (that calculated skin test dilution which produced a summation of erythema diameter of 50 mm). If the D_{50} is >13 the extract contains 100,000 AU/mL, if 11 to 13 it contains 10,000 AU/mL, and if 9 to 11 it contains 1000 AU/mL.

Test and Control Extracts

All allergenic extracts were prepared at baseline by mixing equal parts of the perennial ryegrass extract with one additional extract. Test extracts consisted of perennial ryegrass mixed with either *Helminthosporium interseminatum*, *D. farinae*, or cockroach mix. Control extracts consisted of perennial ryegrass mixed with either shagbark hickory or albumin saline with phenol. The control extracts were mixed at the

beginning of the study and used throughout. Both glycerinated and aqueous (BSP) extracts of each of the above combinations were prepared with the exception of *D. farinae*, which was only available in glycerinated form. Mixed extracts were stored at 2 to 4 °C. At each timepoint aliquots were withdrawn from each mixture vial for in vitro and in vivo analysis.

All extracts and diluents were manufactured by Miles Inc. Allergy Products, Spokane, WA. The specific products are listed below:

Perennial ryegrass, 1:20 (wt/vol) in 50% glycerin

Perennial ryegrass, 1:10 (wt/vol) in BSP

Helminthosporium interseminatum, 1:10 (wt/vol) in 50% glycerin

Helminthosporium interseminatum, 1:10 (wt/vol) in BSP

Dermatophagoides farinae, 30,000 AU/mL in 50% glycerin

Cockroach mix (American, German, Oriental), 1:10 (wt/vol) in 50% glycerin

Cockroach mix (American, German, Oriental), 1:10 (wt/vol) in BSP

Shagbark hickory, 1:20 (wt/vol) in 50% glycerin

Shagbark hickory, 1:10 (wt/vol) in BSP

Albumin saline with 0.4% Phenol

After mixing, the control and test extracts contained 50% of the allergen content specified above, eg. perennial ryegrass, 1:20 (wt/vol), mixed with shagbark hickory, 1:20 (wt/vol), resulted in a final concentration of perennial ryegrass, 1:40 (wt/vol)/shagbark hickory, 1:40 (wt/vol).

Skin Test Control Products

Positive controls: histamine phosphate solution: 1.8 mg histamine base/mL (Center Laboratories, Port Washington, NY) and histamine phosphate solution: 0.1 mg histamine base/mL (Allermed Laboratories, San Diego, CA).

Diluents: 50% glycerin, (Miles Inc. Allergy Products, Spokane, WA) and albumin saline with 0.4% phenol, (Miles Inc. Allergy Products, Spokane, WA).

Relative Potency by RAST Inhibition

Relative potency by RAST inhibition was performed as described in the procedure provided by the United States Food and Drug Administration Center for Biologics Evaluation and Research (CBER).⁹

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli¹⁰ using Mini-PROTEAN II 4% to 15% gradient gels (BioRAD, Richmond, CA). Samples were treated with 1% SDS and 0.04 M dithiothreitol and boiled for three minutes. Gels were stained with Coomassie brilliant blue or transferred to nitrocellulose (0.2 micron, BioRAD, Richmond, CA) and then stained with ISS protein gold (EmProtech, Hyde Park, MA). Blotting was performed electrophoretically in 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.1 using a mini-Trans BlotR Transfer Cell (BioRAD, Richmond, CA).

HPLC

Extracts and mixes were analyzed using a BioRAD Biosil TSK 125 column 300 × 765 mm (BioRAD, Richmond, CA) on a Hewlett Packard model 1090 HPLC analyser. The mobile phase was 0.01 M Tris, 0.9% sodium chloride, pH 7.5 with a flow rate of 0.2 mL/min. Peaks were monitored at 280 nm.

Protease Assay

Extracts were tested for proteolytic activity by incubating 5 μ L in 1% Agarose gels containing 1% casein, pH 7.0 overnight at 37 °C. The plates were fixed with 10% acetic acid. Clearing around the wells indicated proteolytic activity. Activity was scored by the diameter of clear-

ing: >50 mm = +; 21-49 mm = $\pm$,
<20 mm = -.

RESULTS

Relative Potency by Skin Test and RAST

Figure 1 shows the calculated skin test relative potency of the test extracts versus ryegrass/ABS glycerinated and BSP extracts on all test days.

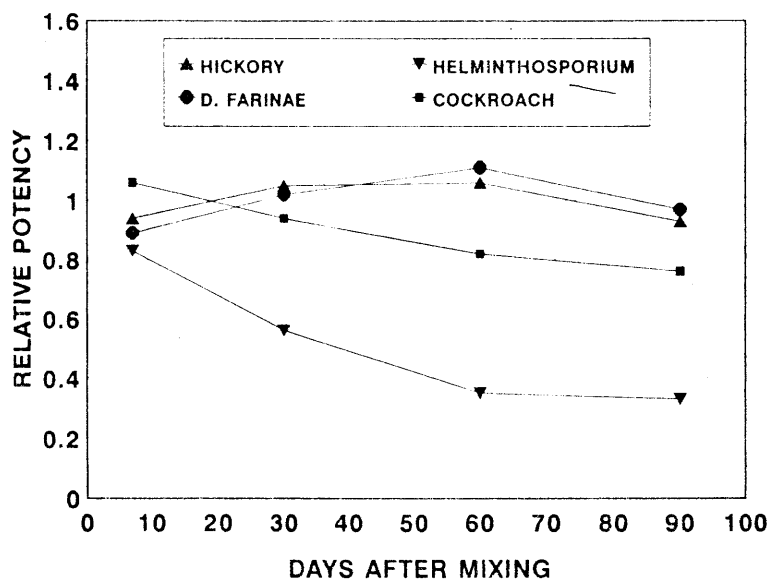
The relative potencies of the extracts were calculated by using the Σ erythema diameter of the skin test reactions. Since two control extracts were used (ryegrass/hickory and ryegrass/ABS), two relative potency determinations were made. Both controls gave the same results so the relative potency between the test extract and ryegrass/ABS is listed. For the glycerin extracts, the ryegrass/*D. farinae* showed no decrease in relative potency compared with the control. The cockroach extract showed a slight decrease in relative potency at day 60 which stayed relatively the same at day 90. The ryegrass/*Helminthosporium* showed the greatest decrease in relative potency first seen at day 30 and becoming greatest at day 60. No further decrease was observed at day 90.

For the BSP extracts, ryegrass/*D. farinae* showed no decrease compared to the control. The ryegrass/cockroach showed a great decrease in relative potency most pronounced at day 90 where it was only 11% as potent as the control. The greatest decrease in relative potency was shown by ryegrass/*Helminthosporium* at day 90 when it was only 4% as potent as the control.

Figure 2 shows the relative potency determinations of the glycerin and BSP extracts and the ryegrass/ABS control at all time points measured by RAST inhibition. The relative potencies measured by RAST are essentially the same as those determined by skin testing.

Figure 3 shows the relative potency determinations of the BSP

A. 50% GLYCERIN



B. BSP

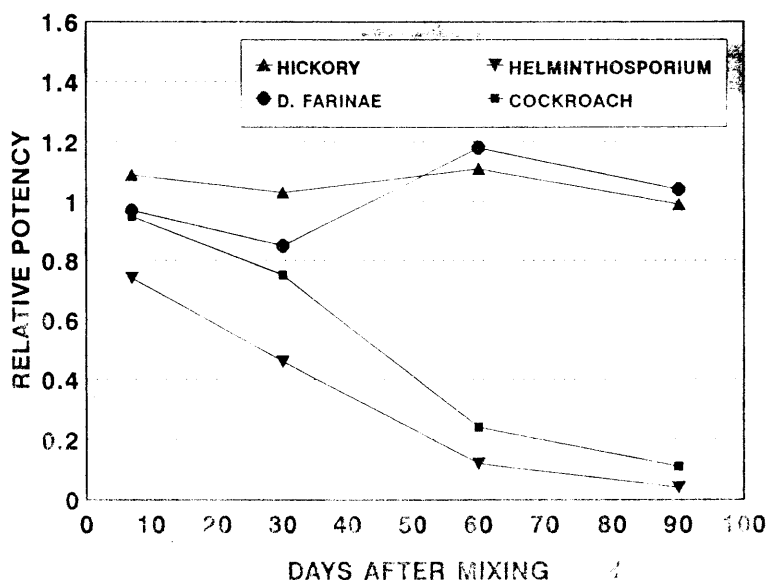
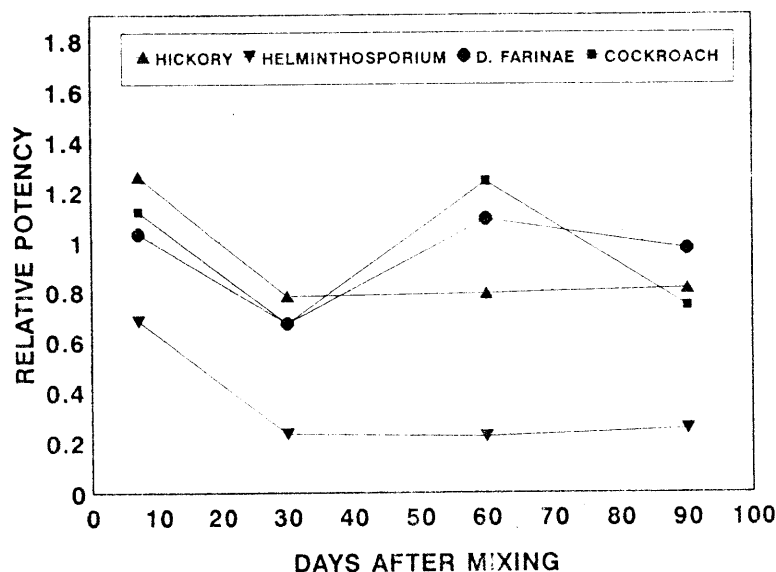


Figure 1. Relative potency by skin test titration of glycerin perennial ryegrass mixed with glycerin test extracts versus glycerin perennial ryegrass mixed with ABS (A) and relative potency of BSP perennial ryegrass mixed with BSP test extracts versus BSP perennial ryegrass mixed with ABS (B).

and glycerin controls. The relative potency of the glycerin and BSP controls were compared at all time

points. The potency of both controls was approximately the same at all time points.

A. 50% GLYCERIN



B. BSP

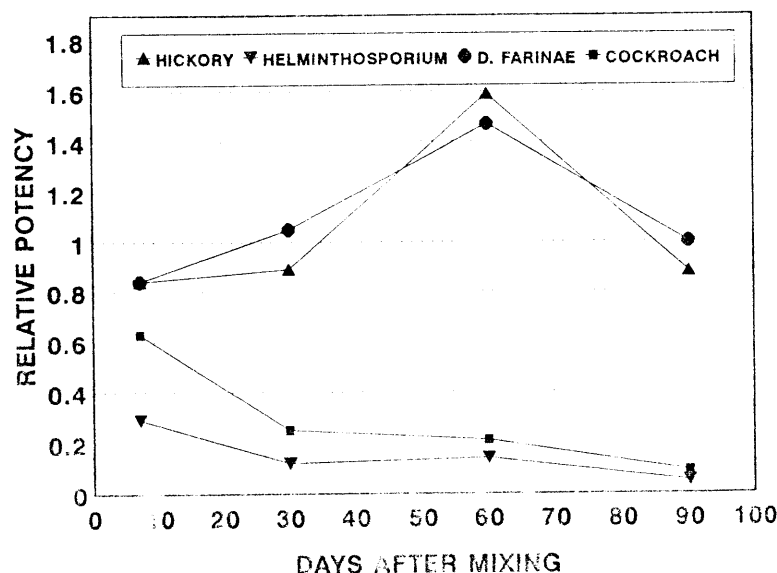


Figure 2. Relative potency by RAST inhibition of glycerin perennial ryegrass mixed with glycerin test extracts versus glycerin perennial ryegrass mixed with ABS (A) and relative potency of BSP perennial ryegrass mixed with BSP test extracts versus BSP perennial ryegrass mixed with ABS (B).

D₅₀ Determinations and AU Calculations

Table 1 shows the D_{50} (dilution pro-

ducing a skin test Σ erythema diameter of 50 mm) and calculated allergy unitage based on three pa-

tients for the test and control glycerin and BSP extracts on all test days. For the glycerinated extracts, the control extracts, ryegrass/hickory and ryegrass/ABS showed little change in D_{50} during the test period. The ryegrass/*D. farinae* and ryegrass/cockroach extracts did not change much over the time period. The ryegrass/*Helminthosporium* showed the greatest decrease in D_{50} from 14.8 at day 7 to 13.8 at day 90.

For the BSP extracts the ryegrass/*D. farinae* showed no decrease in D_{50} during the test period. The ryegrass/cockroach extract showed a decrease in D_{50} , from 14.7 at day 7 to 12.5 at day 90. The ryegrass/*Helminthosporium* showed the greatest decrease in D_{50} from 14.6 at day 7 to 11.7 at day 90. All extracts except those containing cockroach and *Helminthosporium* contained a calculated 100,000 AU/mL ryegrass potency. The cockroach and *Helminthosporium* extracts contained a calculated 10,000 AU/mL.

SDS-PAGE and Blotting

Glycerinated perennial ryegrass mixed equally with albumin saline or glycerinated hickory produced similar SDS-PAGE protein profiles (Fig 4). Similar results were seen with BSP perennial ryegrass equally mixed with albumin saline or BSP hickory but with less intense bands. In both cases major bands were seen at about 70,000 Daltons, 30,000 Daltons and 14,000 Daltons. The HSA band was seen at about 75,000 Daltons. For both glycerinated and BSP perennial ryegrass, mixing with *Helminthosporium* showed a loss of the ryegrass band at 30,000 Daltons.

HPLC

High performance liquid chromatography analysis of perennial rye showed a major peak at 13 to 14 minutes in the albumin saline mix and hickory mix for both glycerin extract and BSP. This peak has a molecular weight greater than

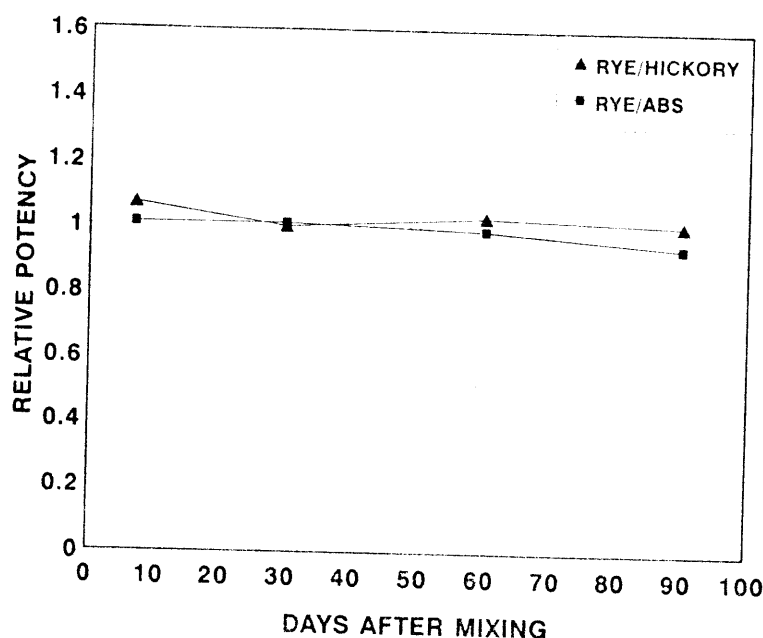


Figure 3. Relative potencies of BSP perennial ryegrass/hickory extract versus glycerin perennial ryegrass/hickory extract and relative potencies of BSP perennial ryegrass/ABS versus glycerin perennial ryegrass/ABS as determined by skin test titration.

Table 1. D50 and Allergy Unit (AU/mL) Values for Control and Test Extracts

	Day 7		Day 30		Day 60		Day 90	
	D50	AU	D50	AU	D50	AU	D50	AU
Glycerin extracts								
Rye/ABS	14.9	100,000	14.8	100,000	14.7	100,000	14.8	100,000
Rye/hickory	14.9	100,000	14.8	100,000	14.7	100,000	14.7	100,000
Rye/Helminthosporium	14.8	100,000	14.2	100,000	13.7	100,000	13.8	100,000
Rye/ <i>D. farinae</i>	15.0	100,000	15.1	100,000	15.1	100,000	15.0	100,000
Rye/cockroach	14.9	100,000	14.4	100,000	14.2	100,000	14.3	100,000
BSP extracts								
Rye/ABS	15.0	100,000	14.7	100,000	14.6	100,000	14.7	100,000
Rye/hickory	14.9	100,000	14.8	100,000	14.8	100,000	14.7	100,000
Rye/Helminthosporium	14.6	100,000	13.9	100,000	12.8	10,000	11.7	10,000
Rye/ <i>D. farinae</i>	15.2	100,000	15.0	100,000	15.1	100,000	15.1	100,000
Rye/cockroach	14.7	100,000	14.2	100,000	13.0	100,000	12.5	10,000

80,000 Daltons. It was not present in the perennial ryegrass/Helminthosporium mixes at day 7 (Fig 5). Lower molecular weight proteins of perennial ryegrass cannot be analyzed because of the interfering Helminthosporium proteins.

Proteolytic Assay

Proteolytic activity was observed as clearing around wells on the casein-agarose gels. Strong clearing was seen with the Helminthosporium and cockroach extracts and minor

clearing with *D. farinae*. There was no clearing with the hickory extract. This is shown in Table 2.

DISCUSSION

Previous studies have shown that mold extracts, particularly those containing Helminthosporium, can have an adverse effect on grass pollen allergenicity when measured by RAST inhibition² or by the ability of such mixtures to produce a measurable IgG response in immunized

mice.¹¹ Cockroach extracts have been reported to contain proteases⁵ that can digest grass extracts.¹² House dust mite extracts also have been reported to contain proteases⁴⁻⁶ that can degrade ragweed extracts.¹³

This study was designed to gather evidence concerning the clinical relevance of proteolytic enzyme digestion of grass pollen allergens by enzyme containing allergenic extracts by determining bioactivity of the extracts. The patients selected were allergic to perennial ryegrass but were not allergic to hickory, Helminthosporium, *D. farinae*, or cockroach extracts. Two control extracts were prepared, ryegrass/ABS and ryegrass/shagbark hickory. The ABS control was used because ABS has no proteolytic activity. The hickory extract was selected because it contained an adequate amount of PNUs (the BSP extract contained 47,000 PNU/mL and the glycerin contained 16,700 PNU/mL) to duplicate what might be seen in a clinical situation when extracts are mixed and did not exhibit proteolytic activity in the casein agarose gels. Glycerin and BSP extracts were both analyzed.

When RAST is used to perform potency studies for stability purposes, a decrease in potency is not considered to be significant unless it is greater than 20%. Using this criteria no significant decrease in ryegrass potency versus the controls was observed in glycerinated extracts at 90 days when mixed with cockroach or *D. farinae*. Helminthosporium did cause a significant decrease first noted at 30 days which became greater at 60 days and was relatively the same at 90 days. For BSP extracts no significant changes were seen with *D. farinae*. Cockroach and Helminthosporium extracts did cause significant ryegrass potency decreases. In all cases the decreases were greater in the BSP than the glycerin extracts suggesting some protective effects by glycerin. Glycerin has been shown to be a superior diluent for single allergen

1 2 3 5 6 7 8 kDa

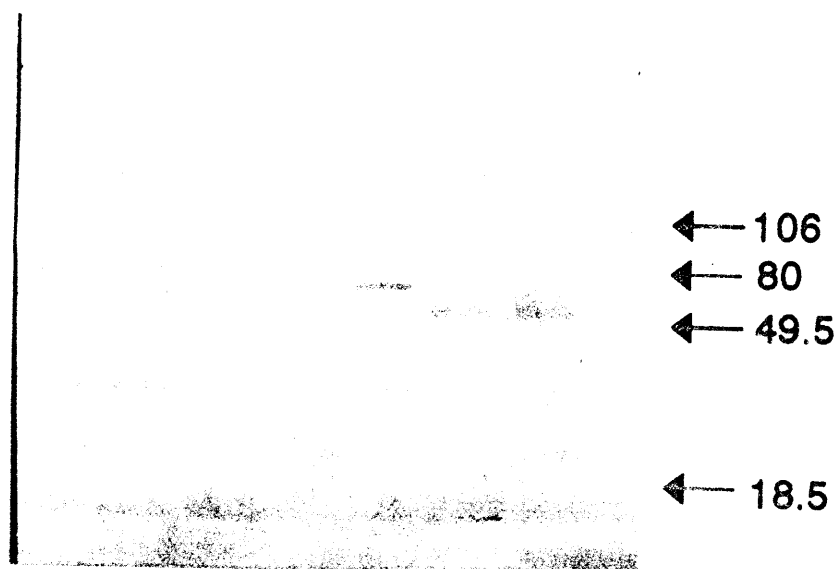


Figure 4. SDS-PAGE of perennial ryegrass mixes in 5% to 15% gradient gels under reducing conditions. Lanes 1-3: glycerinated extracts; lanes 5-7: BSP extracts. Lane 1, perennial ryegrass mixed with albumin saline; lane 2, perennial ryegrass mixed with hickory; lane 3, perennial ryegrass mixed with Helminthosporium; lane 4, empty; lane 5, perennial ryegrass mixed with albumin saline; lane 6, perennial ryegrass mixed with hickory; and lane 7, perennial ryegrass mixed with Helminthosporium.

MOLECULAR SIEVE HPLC OF RYEGRASS AND RYEGRASS/HELMINTHOSPORIUM MIX

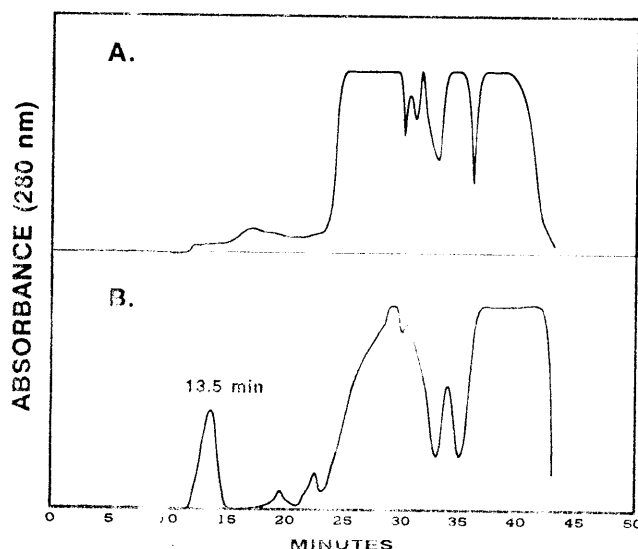


Figure 5. High performance liquid chromatography molecular sieve analysis using a BioRAD Biosil TSK 125 column (300 × 765 nm) of A) perennial ryegrass (BSP) mixed with Helminthosporium (BSP) and B) perennial ryegrass (BSP). Peaks were monitored at 280 nm.

Table 2. Proteolytic Assay Results for Allergenic Extracts

	Glycerin	BSP
Helminthosporium	+	+
Cockroach	+	+
<i>D. farinae</i>	±	N/A
Ryegrass	-	-
Hickory	-	-

extracts, as those containing at least 50% glycerin are more stable than those prepared using other diluents.¹⁴

When using skin testing to compare relative potency, the results were similar to those obtained by RAST. No change was noted for *D. farinae*. Cockroach BSP showed an 89% decrease in relative potency by skin test and a 91% decrease by RAST at day 90. Cockroach glycerin extract showed a 24% decrease by skin testing and a 26% decrease by RAST at day 90. The Helminthosporium extracts showed a similar relationship when examined by RAST and skin testing. The Helminthosporium BSP extract showed a 96% decrease in potency at day 90 by skin testing and a 95% decrease by RAST. For the glycerin extract the decreases were 67% by skin testing and 75% by RAST.

A proteolytic assay showed significant proteolytic activity was present in the Helminthosporium, cockroach, and *D. farinae* extracts but none was present in the control shagbark hickory extract. Several *D. farinae* extracts were examined using the proteolytic assay and significant differences were noted in proteolytic activity for the different extracts. The one used in this study exhibited moderate proteolytic activity. The SDS-PAGE assays and the HPLC analysis showed a decrease in one perennial rye protein after mixing with Helminthosporium which confirmed earlier reported observations. No loss of SDS bands was seen with the cockroach or *D. farinae* extract mixes.

Many allergenic extracts are standardized in allergy units (AUs) which are assigned to extracts by

determining D_{50} s in very sensitive patients.⁸ Extracts labeled at 100,000 AU/mL elicit D_{50} s of between 13 and 15. Those labeled at 10,000 AU/mL have D_{50} from 11–13. To accurately assign an unknown extract a given potency in allergy units, at least 15 to 20 sensitive patients must be tested. In our study we tested only three patients to each extract at each timepoint. As expected, the control extracts and the mixes tested at day 7 exhibited D_{50} s that were in the 13–15 range helping to confirm the fact that the ryegrass extract did contain 100,000 AU/mL (even when diluted 50% by a control or test extract).

The decrease in extract potency caused by mixing enzyme containing extracts with perennial ryegrass is approximately the same when determined by relative potency skin test measurements and RAST. In the worst case (ryegrass/*Helminthosporium* in BSP) the D_{50} was 14.6 at day 7 and decreased to 11.7 at day 90. Assuming this D_{50} value is truly predictive of extract potency, the extract at day 90 still contained approximately 10,000 AU/mL of ryegrass. The loss of activity may be due to the decrease in the protein corresponding to the band on SDS-PAGE at around 30,000 Daltons or the HPLC peak at 13.5 minutes. The remaining allergenic activity suggests not all of the allergens are susceptible to the proteolytic enzymes. Extracts of 10,000 AU/mL are routinely used to treat patients allergic to mites, cats, and other allergens with good therapeutic responses. The volumes administered must be greater with these extracts than with ones containing 100,000 AU/mL, but if appropriate changes in doses are made one would expect a 10,000 AU/mL ryegrass extract to be effective for immunotherapy. Grass pollen extracts are so potent that even relatively large decreases in relative potency can result in an extract that is still

as potent as many other effective extracts. This may help to explain why such mixes, which have been widely used in clinical practices for many years, do seem to provide therapeutic efficacy (at least anecdotally).

More studies must be performed to determine the exact clinical relevance of these observations. Studies have shown that changing to a freshly prepared extract can be associated with severe, even fatal, anaphylactic reactions if the dose of the new one is not sufficiently decreased.¹⁵ The enzyme induced degradation of pollen allergenicity might, in part, explain some of these untoward reactions. Our studies have shown that enzymatic activity varies even between lots of the same extract. It is impossible to know for a specific extract how much proteolytic activity is present so, for the sake of safety, the physician should assume that mold extracts could contain several enzymes. Until the potency of extract mixes can be routinely measured for clinical purposes, the allergist might be well advised to not mix extracts containing proteolytic enzymes, particularly *Helminthosporium*, with grass and perhaps other pollen extracts.

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